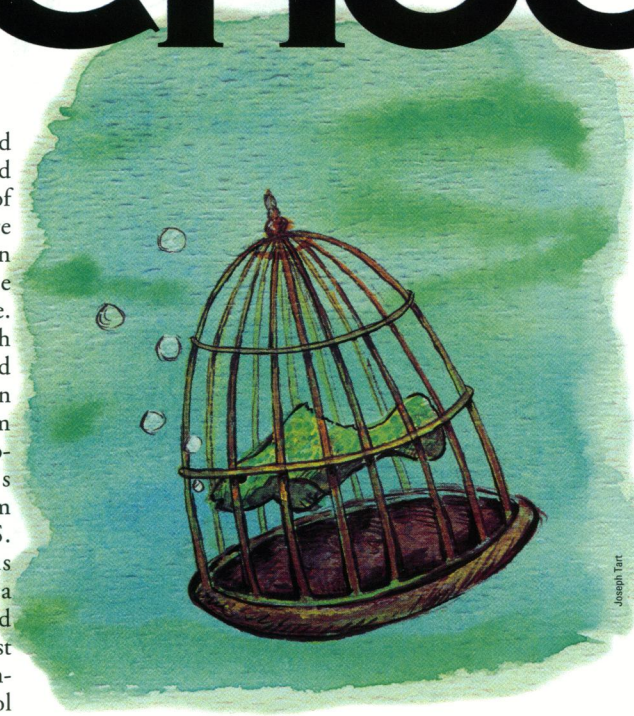
SENTINEL SCHOOL



A century ago, coal miners carried caged canaries into underground mines to alert them to the presence of carbon monoxide gas. Canaries are more sensitive to many gases than humans, and if the birds died, the miners knew to evacuate the mine. Today, organizations concerned with water quality, including the EPA and the U.S. Army, are searching for an environmental sentinel to alert them to the hazards of potentially carcinogenic chemicals in the nation's waters. NIEHS chemist Jim Burkhart, working with the U.S. Army and scientists at various research institutes, is developing a recoverable bacteriophage inserted into a transgenic fish that may be just what they are looking for. This combination promises to provide a tool that can be used in both laboratory and field studies to make a rapid determination of genetic health hazards to human and animal populations.

There are any number of pathogens or chemicals which, if present in a body of water in high enough concentrations, will kill fish and cause humans to get sick. Scientists have established safe levels of tolerance for most of these agents and governments have incorporated these levels into the appropriate regulations. But there are many other chemicals that have far more subtle effects on living organisms, such as inducing genetic mutations that may lead to cancer and other diseases over a lifetime of exposure. Science does not have a clear understanding of dose–effect relationships for most of these chemicals and therefore few standards have been set.

There are few methods available that can be used to assess genetic hazards for aquatic species, or that focus on the study of particular gene mutations as they occur in DNA *in vivo*. And even where scientists have been able to demonstrate induced genetic effects for aquatic species, they have not been able to compare that to the situation for humans. There are enough differences in exposure, metabolism, and gene expressivity to make correlations between fish and humans a hazardous enterprise.

Added to the problem of cross-species comparisons are the sheer numbers of observations required for studies of mutagenesis in aquatic species and mammals. The cost, time, and physical requirements of such studies can make them impractical, if not impossible, in many circumstances. "Take the example of a base pair substitution that can alter a gene's function," Burkhart says. "That event happens at a very low *in vivo* frequency . . . and requires a large number of observations to detect mutations (often in the tens of thousands) which means that a large number of animals must be used. And it requires unambiguous detection of mutants among the total number of genes analyzed. The technology does not exist at this time to realistically meet these experimental conditions with natural chromosomal gene sequences, especially in conditions where there has been little genetic analysis at the DNA level."

Along with the need to use fewer animals is the need to use phylogenetically lower species than mammals. Space requirements, cost, and ethical standards argue against the use of large numbers of rodents or higher animals for mutagenic studies. As such, there is a need for a model that can be used to quickly and easily detect environmentally induced mutation in aquatic species, and to accurately correlate that data with potential hazards to mammalian species including man.

The Transgenic Approach

Scientists have recognized that the complexities involved in studies of mutagenesis across species could be reduced by using the same gene marker for mutation in a variety of species. A target for mutation analysis that is independent of any requirement for expression, growth, or selection in tissue could be introduced into different host species. The resulting transgenic animals could be exposed and the transgene later recovered to observe mutations. Comparison of mutation rates could then be made between somatic tissues and correlated with other endpoints. Identical transgenes introduced into rodents, fish, or cultured human cells would simplify the cross-species comparison.

NIEHS researchers have paved the way

in the development of transgenic mouse models for detection of mutations and understanding of how alterations of certain critical genes may be involved in disease. Beginning in 1991, Burkhart, in collaboration with H. V. Malling of the NIEHS, Rebecca Van Beneden, then of Duke University, and colleagues set out to develop an *in vivo* mutation detection system using transgenic fish with a chromosomally integrated and recoverable bacteriophage, named ΦX174.

"ΦX174 is a bacteriophage that has markers for mutation that are already well-characterized," Burkhart says. "It is not normally expressed in eukaryotes, so we don't have the problem of different phenotypic expressions across individuals or species. It's very small, which permits the insertion of many copies in the host genome with less chance of chromosomal disruption than larger constructs."

Burkhart has chosen two species of fish for transgenic development, the medaka (*Oryzias latipes*), a freshwater species, and the mummichog *(Fundulus heteroclitus),* an estuarine species. Both are ideal for development as transgenic fish because of their small size, manipulable eggs, and short embryogenesis. Both have been used extensively for studies of chemical carcinogenesis and toxicology.

The methodology employed by Burkhart involves linearizing the circular bacteriophage DNA and ligating it to form a ladder of ΦX174 catenates. Low numbers of phage sequences per host genome contribute to low recovery of the phage. Therefore, in the production of transgenic animals, the copy number is increased by catenating the DNA. A solution of either a single gene copy or catenated ΦX174 DNA is physically injected or electroporated into fish eggs, which are then allowed to hatch. The fish are then analyzed for integration of the transgene and are used to establish a transgenic line. At the chosen stage in the fishes' development, the vector DNA sequence is recovered from the hosts' genomic DNA. The phage DNA is transfected into a specialized strain of *E. coli* for packaging, then plated with various selective indicator bacteria to determine the total number of progeny phage recovered and the number of mutants. In experiments run over the past five years, Burkhart and his colleagues have successfully produced transgenic medaka, mummichog, and mice, as well as cultured human, hamster, mouse, and fish cells.

In the development of transgenic animals, scientists have observed that methylation, a natural mechanism of DNA modification, can be a major obstacle to comparisons of DNA sequences across different species. Methylation varies as a function of tissue, sex, age, and parental origin. Any molecular approach that responds to natural methylation, independent of actual mutation, would be seriously limited in its utility for comparing data between different species. Studies with transgenic mice using ΦX174 showed extensive methylation after two generations, which resulted in reduced recovery of the phage. The problem was solved for mice by producing a new *E. coli* strain for recovery of the methylated phage DNA. Fortunately, this method has also proven effective for fish, allowing for a correlation to be made between species without the unknown contribution of variable methylation.

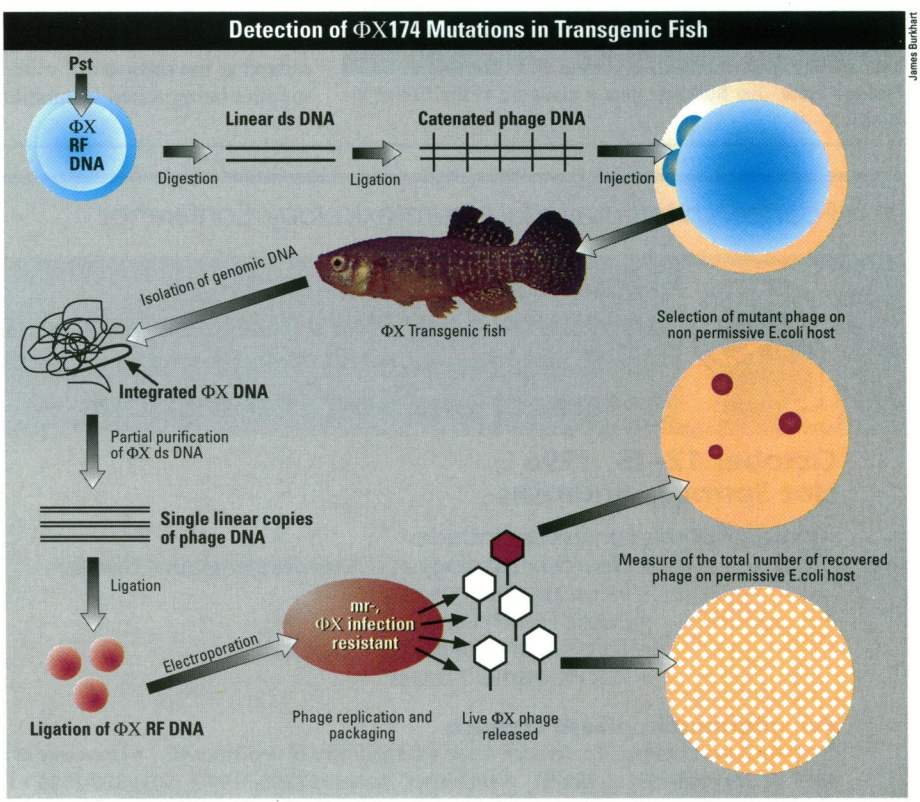
Steve McCaw, Image Associates, Inc.

Mutant mummichogs. Transgenic *Fundulus heteroclitus* may indicate risks to human health.

Spontaneous mutations occur among phages recovered from the DNA of untreated transgenic animals, as well. Burkhart has measured the range of spontaneous mutation frequencies in the transgene to be between 1.5×10^{-7} and 3.7×10^{-7} for fish, mice, and cultured cells.

Initial experiments have been run exposing transgenic mice and fish to mutagens including *N*-ethyl-*N*-nitrosourea (ENU) and polycyclic aromatic hydrocarbons (PAHs), pollutants commonly found in U.S. waters. Burkhart has measured the induced mutation frequencies for the transgene and is assembling data to allow comparison across species.

"Taken together, our current results indicate that ΦX174 may be useful as an identical gene target in aquatic species, in laboratory animals, and in cell cultures with a potential for comparative mutagenesis studies in both basic and applied research," Burkhart says. "The advantages of this approach are that it measures mutagenic effect at the DNA level, it is not limited by gene expression to a cell type, and

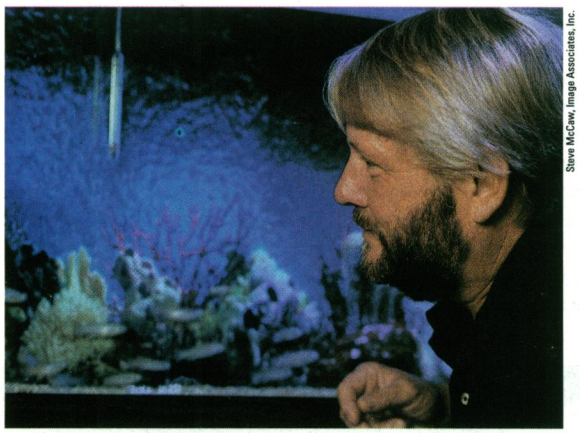


it has target specificity combined with a numerical power not practically available in other systems."

Fish out of Water

The transgenic approach to detection of mutations in aquatic species could have direct applications in a number of areas. Gary Boorman, deputy director of the Office of Special Programs in the NIEHS's Laboratory of Experimental Pathology, sees great use for Burkhart's system in understanding the potential toxicity of chemicals used to disinfect drinking water.

"Disinfection of drinking water is one of the greatest public health advances of this century," Boorman says. "But in the last few years, studies have shown that when used at 100,000-fold concentrations to normal exposure, byproducts of these disinfectants cause cancer in mouse liver tumors. If disinfectants are going to protect human health, they are going to have some toxicity. The question is what are the best levels or chemicals to protect human health. [Burkhart's] model will be helpful in giving comparative toxicities and at more sensitive levels than is presently available. With the proper knowledge, utility engineers could switch the balance of disinfectants to make more or less of different classes of byproducts."



Steve McCaw, Image Associates, Inc.

Fisheye view. Jim Burkhart gets a close-up of the fish of his labors.

Scientists at the Army's Research Institute of Environmental Medicine are currently cosponsoring research with the NIEHS on the use of the transgenic fish approach for assessing human health hazards in aquatic environments. They are hoping that such a system can be used to evaluate hazardous waste sites, effluents, and drinking water supplies that might be used by the military. They are particularly excited at the prospect of water supplies being tested in mobile units with this system. "We want quicker, cheaper, and more relevant ways to test complex environmental mixtures," says Hank Gardner, director of the U.S. Army's Biomedical Research and Development Laboratory. "We've been using standard mutagenicity assays, which are *in vitro* cell-based assays. The opportunity with [this] model is that it provides *in vivo* data and that can be related to mammalian species as well."

Burkhart is quick to point out that studies with the ΦX174 bacteriophage have provided parallel mutation data among fish and mammals for only a limited number of chemicals and target genes. Further research will be needed to verify the system's efficacy for a larger number of genes and with a variety of environmental agents. Gardner, for one, is hopeful about the prospects. "No model can predict with absolute certainty the risk to human health," he says. "But [this] approach offers the best opportunity that I know."

John Manuel

Suggested Reading

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